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# Biological Control of Postharvest Rots in Fruits Using 'Debaryomyces hansenii'

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Abstract: The present invention relates to the biological control of postharvest diseases in fruit. More particularly, the invention relates to a method for biologically controlling postharvest rots on fruits using a strain of Debaryomyces Hanseii, Postharvest diseases of fruit cause 15 to 25% losses yearly in the fruit industry worldwide. Fungicides, the major weapon in combatting these diseases, are often ineffective and pose hazards to humans and the environment. Therefore, a critical need exists for new methods to control postharvest rots on fruits using a strain of Debaryomyces hansenii. Postharvest diseases of fruit cause 15 to 25% losses yearly in the fruit industry worldwide. Fungicides, the major weapon in combatting these diseases, are often ineffective and pose hazards to humans and the environment. Therefore, a critical need exists for new methods to control postharvest diseases.

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# Biological Control of Postharvest Rots in Fruits Using *Debaryomyces hansenii*

## Background of the Invention

### Field of the Invention

The present invention relates to the biological control of postharvest diseases in fruit. More particularly, this invention relates to a method for biologically controlling postharvest rots on fruits using a strain of *Debaryomyces hansenii*, (Zoph) van Rij, "*D. hansenii*."

### Decription of Prior Art

Postharvest diseases of fruit cause 15 to 25% losses yearly in the fruit industry worldwide. Fungicides, the major weapon in combatting these diseases, are often ineffective and pose hazards to humans and the environment. Therefore, a critical need exists for new methods to control postharvest diseases.

Recently, it has been shown that the postharvest treatment of fruit with antagonistic microorganisms is an effective approach to the control of postharvest rots. Remarkable success was shown in the

control of brown rot in peaches caused by Monilinia fructicola (Wint.) Honey with Bacillus subtilis. Pusey et al. [Plant Dis. 86:753-756 (1986)]. De Matos was able to reduce mold incidence from 35% to 8% when a species of Trichoderma was inoculated with Penicillium digitatum into lemon peel. De Matos, Ph.D. Dissertation, University of California, Riverdale, (1983). Singh and Deverall demonstrated biocontrol with bacterial antagonists to the citrus pathogens Alternaria citri Pierce, Geotrichum candidum link. ex Pers., and P. digitatum. Singh et al. [Trans. Br. Mycol. Soc. 83:487-490 (1983)]. Dipping wounded citrus fruit in suspensions of bacterial cells, particularily a strain of Bacillus subtilis (Ehrenber) Cohn, delayed decay by the three rot pathogens.

## Summary of the Invention

We have discovered a new strain of D. hansenii that is highly effective in controlling a variety of fruit-rot pathogens which affects several species of fruit. Two isolates of the new strain have been deposited with the culture collection at The Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Illinois 61604, under the acquistion numbers NRRL Y-18313 and NRRL Y-18314. Progenies of the strain will be available during the pendency of the patent application to one determined by the Commissioner of Patents

and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restrictions on the availability of progenies of the strain to the public will be irrevocably removed upon the granting of the patent of which the strain is the subject.

Accordingly, it is an object of the present invention to provide a novel biological control agent which is safe and highly effective to control a variety of postharvest diseases in a variety of fruits.

It is also an object of the invention to provide a method of biologically controlling postharvest diseases in fruits which elminates the use of fungicidal treatments.

In accordance with our invention, fruits are subjected to a an aqueous suspension comprising an isolate of D. hansenii having the identifying characterics of an isolate selected from the group consisting of NRRL Y-18313 and NRRL Y-18314. In effect, the organisms mutiply and occupy the surfaces of wounded fruit, thereby perventing infection by fruit-rot pathogens.

## Detailed Description of the Drawings

Isolates NRRL Y-18313 and NRRL Y-18314 were obtained from the surface of citrus fruits by repeatedly washing the fruit with water. The organisms are thereafter plated and grown on any nutritionally

rich meduim sufficient to support growth of the organisms. Preferably, the medium is either nutrient yeast dextrose agar (NYDA) or yeast-malt extract agar (YM).

Isolates NRRL Y-18313 AND NRRL Y-18314 have the following indentifying characteristics: Colonies are cream white, slighly raised, shiny, round and smooth. No pseudohyphae were observed. No ascospores were produced after one week on Corn Meal agar, V-8 Juice agar, YM or acetate. On solid YM, cells are unicellular in liquid culture after one day. Small globose cells are observed mainly in chains or clusters, many with one bud.

Biochemical and physiological tests of the isolates were as follows:

| Carbon Assimilation: | NRRL Y-18314 | NRRL Y-18313 |
|---|---|---|
| Glucose | + | + |
| Galactose | + | + |
| L-sorbose | + | + |
| Maltose | + | + |
| Sucrose | + | + |
| Cellobiose | + | + |
| Trehalose | + | + |
| Lactose | - | - |
| Melibiose | + | - |
| Raffinose | + | + |
| Melezitose | + | + |
| Inulin | + | + |
| Soluble Starch | w | w |
| D-xylose | + | + |
| L-arabinose | + | + |
| D-arabinose | + | + |
| D-ribose | + | + |
| L-rhamnose | + | w |
| D-glucosamine | + | w |

| Carbon Assimilation (cont.): | NRRL Y-18314 | NRRL Y-18313 |
|---|---|---|
| Ethanol | w | w |
| Erythritol | w | - |
| Glycerol | + | + |
| Adonitol (Ribitol) | + | + |
| Dulcitol (Galactitol) | + | + |
| D-mannitol | + | + |
| D-sorbitol (glucitol) | + | + |
| a-methly-D-glucoside | + | + |
| Salicin | + | + |
| Inositol | - | - |
| Lactic acid | w | + |
| Citric acid | + | + |
| Succinic acid | + | + |

| Nitrogen assimilation: | NRRL Y-18314 | NRRL Y-18313 |
|---|---|---|
| $NH-NO_3$ | + | + |
| $KNO_3$ | + | + |
| $NO_2$ | w | w |
| Ethylamine | + | + |

| Fermentation: | NRRL Y-18314 | NRRL Y-18313 |
|---|---|---|
| Glucose | + | + |
| Galactose | w | + |
| Maltose | - | - |
| Sucrose | + | + |
| Lactose | - | - |
| Raffinose | - | - |
| Melibiose | - | - |
| Inulin | w | - |
| Cellobiose | - | - |
| Melezitose | - | - |
| Starch | - | - |
| Trehalose | - | - |

w = weak

Growth of the organisms is effected under aerobic conditions at any temperature satisfactory for growth of the organisms, i.e. from about 10 °C to about 30 °C. The preferred temperature range is about 20 °C to 25 °C. The pH of the nutrient medium is about neutral, ie. 6.7 to 7.2. The incubation time is that time necessary for the organisms to reach a stationary phase of growth, preferably, from about 40 to 60 hours.

Isolates NRRL Y-18313 and NRRL Y-18314 may be grown in any conventional shake flask for small fermentation runs. For large scale operations, it is convenient to carry out the culture in a fermentation tank, while applying agitation and aeration to the inoculated liquid medium. Following incubation, the organisms are harvested by conventional sedimentary methodology, i.e. centrifugation or filtering. Cultures are stored on silica gel and frozen until use.

Isolates NRRL Y-18313 and NRRL Y-18314 are useful to control a variety of fruit-rot pathogens which causes postharvest diseases in fruits. Exemplary species of fruit-rot pathogens include, but are not limited to, Penicillium italicium Wehmer, Penicillium digitatum, Botyrtis cinerea, Rhizopus stolonifer, Geotrichum candidum, Penicillium expansum, and Alternaria alternata.

The organisms of the invention are useful to control postharvest diseases in a variety of fruit including, but not limited to, all cultivars of citrus fruits, grapes, apples, pears, tomatoes,

persimmons and the like. Suitable citrus fruits include, but are not limited to, grapefruits, oranges, lemons and the like.

The organisms of the invention are preferably applied to the fruits in suspension with water. When grown in a liquid medium, the organisms may be applied in suspension with the liquid medium. Suspensions of the organisms of the invention may also include conventional additives such as surfactants and wetting agents to enhance the effectiveness of the organisms.

Concentrations of suspensions useful in the invention are any concentrations which inhibits the development of the targeted fruit-rot pathogen when applied to the fruit. As will be obvious to one skilled in the arts, effective concentrations may vary depending upon such factors as (1) the type of fruit; (2) the ripeness of the fruit; (3) the concentration of pathogens affecting the fruit; (4) the type of wound on the fruit; (5) temperature and humidity; and (6) the age of the fruit-rot pathogen. Exemplary concentrations ranges from about $1 \times 10^4$ to $1 \times 10^9$ CFU/ml, most preferably, from about $1 \times 10^7$ to $1 \times 10^9$ CFU/ml. For purposes of the invention, the abbreviation "CFU" is used herein to designate "colony forming units."

The organisms of the invention may be applied to fruits using conventional methods such as dipping, spraying or brushing. In addition, the organisms of the invention may be incorporated into waxes, wraps or other protecting coatings used in processing the

fruits.

The fruits may be treated anytime before or after harvest. Typically, the preferred time of treatment is after harvest and prior to storage or shipment. In the case of some grapes, the preferred time of treatment is before harvest.

It is within the compass of the invention to treat the fruits with either of isolates NRRL Y-18313 or NRRY Y-18314 alone, or in combination. The organisms may also be used in combination with other control agents useful to inhibit the development of fruit-rot pathogens on fruits. When used, these agent should be use in an amount, as readily determined by one skilled in the arts, which will not interfere with the effectiveness of the organisms of the invention.

The following examples are intended to further illustrate the invention and not to limit the scope of the invention as defined by the claims.

Example I

The effectiveness of D. hansenii NRRL Y-18314 was evaluated using the following seven citrus cultivars: grapefruit (Citrus paradisi Macf. cv 'Marsh Seedless'); 'Shamouti' and 'Valencia' orange (C. sinensis Osbeck); lemon (C. lemon L. Burm 'Eureka'); Temple orange (Tanger hybrid, C. reticulata X C. sinensis); Kumquat (Fortunella

margarita); and pummelos, (C. grandis). Fruit rot pathogens tested included Penicillium digitatum, Penicillium italicum and Geotrichum candidum Link. ex Pers., fungi responsible for the postharvest diseases green-mold, blue-mold and sour-rot, respectively.

A biologically pure culture of isolate NRRL Y-18314 was obtained using the following procedures: The surface of lemons was washed by placing the fruit in a 600 ml beaker containing 200 ml of sterile water. The beakers containing the fruit were placed on a rotary shaker at 100 rpm for 10 minutes. One tenth ml of the wash water was then spread on a NYDA plate and allowed to incubate for 24 hours before colonies were selected. The same fruit received three separate washings and the same procedures were followed. Appearing colonies were isolated and purified using standard purification techniques. All cultures were stored on silica gel in a freezer until use.

Isolate NRRL Y-18314 was grown in flasks containing nutrient yeast dextrose broth (NYDB) on a reciprocal shaker at 30 $^{0}$C for 48 hours. The culture was centrifuged at 7000 rpm for 10 minutes and the resulting pellet was suspended in water at various concentrations. Concentrations of the aqueous suspensions were adjusted on a spectrophotometer.

Freshly harvested fruit was wiped with 95% ethanol and placed on

moist paper in 50 x 100 x 15 cm plastic trays, 24 fruits per tray. Two to four conical wounds, 3mm deep, were cut in the fruit peel. The wounds were brushed with an aqueous suspension of NRRL Y-18314. Concentrations of the aqueous suspensions ranged from $1 \times 10^5$ to $1 \times 10^{10}$ CFU/ml. One to two hours later, 20 µl of an aqueous spore suspension of the targeted pathogen, $1 \times 10^4$ spores/ml, were pipetted into the wounds. Control fruits were inoculated with aqueous spore suspensions of the targeted pathogen only. Following incubation, the trays were covered with high density polyethylene sleeves and kept at room temperature for several days.

The number of inoculated sites on which decay developed was determined daily. Each treatment in each experiment consisted of at least 3 replicates of 6 fruits, 24 to 75 inoculation sites per treatment. Each experiment was repeated at least twice.

Results were analyzed and are recorded in Tables I, II, and III below.

As shown in Table I, D. hansenii, isolate NRRL Y-18314, was highly effective in inhibiting Penicillium digitatum decay on citrus fruit in all cultivars tested. The effectiveness of NRRL Y-18314 varied depending upon the sensitivity of the cultivar to the decay. When compared to its effectiveness on grapefruit, isolate NRRL Y-18314 was

more effective on pummelo fruit but less effective on temple, lemon, orange, or kumquat fruits.

Table II shows that isolate NRRL Y-18314 was effective in inhibiting Pencillium italicum decay on grapefruit, oranges and other citrus fruit cultivars. As in the case of Pencillium digitatum, NRRL Y-18314 more effectively controlled Pencillin italicum in grapefruits than in oranges. NRRL Y-18314 was also effective in inhibiting the development of Geotrichum candidum in citrus fruits. However, as shown in Table III, Geotrichum candidum was controlled to a lesser extent than the Penicillia decays, particulary in lemons.

Example II

The ability of D. hansenii NRRL Y-18314 to inhibit Rhizopus rot development in grapes was demonstrated.

A biologically pure culture of NRRL Y-18314 was isolated and purified as described in Example I.

NRRL Y-18314 was incubated in 100 ml of NYDB in 250 ml Erlenmeyer flasks on a rotary shaker (100 rpm) at 28°C for 48 hours. Freshly harvested grapes of the Perlette and Thompson Seedless cultivars were dipped monentarily in a suspension of the organism in NYDB. The berries were treated as whole clusters with non-injured berries, as injured berries which had been removed from the stems by pulling and

thereby causing a wound, or as injured single berries wounded by piercing non-injured berries with a needle. Control berries were dipped in sterile NYDB only.

One to two hours after the berries had been dipped in the suspension, the berries were dried and thereafter inoculated by dipping in an aqueous suspension containing spores of the targeted pathogen at a concentration of $1 \times 10^4$ spores/ml. Alternatively, the berries were inoculated by placing a single decayed berry in the center of a group of non-injured berries, i.e. "nesting." The treated berries were placed in polyethylene-covered cartons and held at room temperature for 5 days. Whole treated clusters were placed directly in commercial shipping cartons.

Decay incidence was determined by counting the number of infected berries. Each treatment in each experiment consisted of at least three replicates of 20 berries or four replicates of five intact clusters placed in half of a shipping carton.

The results were analyzed and are recorded in Table IV.

As shown in Table IV, D. hansenii NRRL Y-18314 was effective in reducing Rhizopus rot in both injured and non-injured grape berries. Reduction of decay was most pronounced in berries that were not injured prior to inoculation and inoculated by nesting.

Example III

The effectiveness of isolate of D. hansenii NRRL Y-18314 to inhibit Botrytis cinerea and Pencillium expansum rot was tested on apples.

Golden Delicious apples were washed with 2% sodium hypochlorite to surface sterilize the fruit. After air drying, the apples were placed on styrofoam trays in plastic trays with lids. Water (100 ml) was added to each tray for humidity. The apples were wounded using a needle. Wound size was 4mm wide by 5mm deep. Three-day old shake cultures of NRRL Y-18314 growing on NYDB at a 1 X $10^9$ CFU/ml concentration was added to the wounds, 50 µl/wound. Apples were allowed to air dry. Thereafter, an aqueous suspension of Botrytis cinerea or Penicillium expansum spores, 1 x $10^4$ spores/ml, were added to the wounds, 20 µl/wound. Controls were inoculated with water only.

Measurements of infected areas were taken 5, 7, 9 days after inoculation. Results were analyzed and recorded in Tables V and VI below.

NRRL Y-18314 effectively controlled both Botrytis cinerea and Penicillium expansum rots in apples. As shown in Table V, total protection against Botrytis cinerea occurred in treated apples up to

about 7 days after inoculation, with only small lesion development after nine days. Protection against Pencillium expansum was to a lesser extent than against Botrytis cinerea. Nevertheless, Table VI clearly shows that apples treated with NRRL Y-18314 had a significant decrease in the development of Pencillium expansum when compared to the untreated controls.

Example IV

The effectiveness of D. hansenii NRRL Y-18314, to inhibit Penicillium digitatum on grapefruit was compared to the effectiveness of eight previously identified isolates of D. hansenii.

The eight isolates were obtained from the American Type Culture Collection, hereinafter referred to as "ATCC," located at 12301 Parklawn Drive, Rockville, M.D. 20252, USA. Identification of the isolates tested were as follows: ATCC 18538, ATCC 20220, ATCC 36239, ATCC 34022, ATCC 36239, ATCC 9367, ATCC 36767, and ATCC 18107.

Each isolate of D. hansenii tested was incubated in NYDB liquid medium at $28^0$C for 48 hours. Following centrifugation, the resulting pellets were washed twice with water and thereafter suspended in water. Concentrations of the aqueous suspensions ranged from $1.3 \times 10^7$ to $1.3 \times 10^9$ CFU/ml.

The surface of the grapefruit was sterilized with 95% ethanol and

placed on moist paper in 50 x 100 x 15 cm plastic trays, 24 fruits per tray. Thereafter, the surface of the fruit was wounded using a needle. Two to four conical wounds, 3 mm deep, were cut in the fruit peel. An aqueous suspension of an isolate of D. hansenii was brushed onto the surface of the wound. Each isolate was tested on 48 sites of inoculations. One to two hours later, an aqueous suspension of Penicillium digitatum, $1 \times 10^5$ spores/ml, was added to the wounds, 20 µl/wound. Controls were inoculated with water only.

The percent of fruit infection was recorded 7 days after inoculation. The data was analyzed by analysis of variance and means were separated by Duncan's Multiple Range Test. Different letters are significant at a 1% level. The results are recorded in Table V.

D. hansenii NRRL Y-18314 clearly exhibited superior control of Penicillium digitatum when compared to prior identified isolates of D. hansenii. After seven days of inoculation, total protection occurred in grapefruits inoculated with NRRL Y-18314 while as much as 25 to 65% infection occurred in fruits inoculated with isolates obtained from the ATCC (see Table VII).

It is understood that modifications and variations may be made to the foregoing disclosure without departing from the spirit and scope of the invention.

TABLE I

Relative effectiveness of *Debaryomyces hansenii* (NRRL Y-18314) in inhibiting *Penicillium digitatum* decay of different citrus cultivars.

| Citrus cultivar | Antagonist | Incubation time (days) 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|
| | | Percent Infection[a] | | | |
| Grapefruit (72) | NRRL Y-18314 | 0 | 2 | 6 | 11 |
| | Control | 90 | 97 | 100 | 100 |
| Orange, 'Shamouti' (42) | NRRL Y-18314 | 0 | 3 | 10 | 17 |
| | Control | 93 | 100 | 100 | 100 |
| Orange, 'Valencia' (42) | NRRL Y-18314 | 2 | 4 | 8 | 17 |
| | Control | 90 | 94 | 97 | 100 |
| Lemon (42) | NRRL Y-18314 | 0 | 2 | 10 | 15 |
| | Control | 98 | 100 | 100 | 100 |
| Temple (48) | NRRL Y-18314 | 2 | 4 | 10 | 14 |
| | Control | 95 | 96 | 99 | 100 |
| Pummelo (24) | NRRL Y-18314 | 0 | 0 | 2 | 2 |
| | Control | 83 | 90 | 92 | 96 |
| Kumquat[b] (150) | NRRL Y-18314 | 4 | 8 | 12 | - |
| | Control | 19 | 23 | 37 | - |

[a] Number of inoculation sites per treatment is indicated in parentheses under the cultivar's name.

[b] Whole fruits were used without artificial inoculation. The fruit was dipped momentarily in a 48 hr-old liquid culture of the NRRL Y-18314. NYDB was used as control.

TABLE II

Inhibition of *Penicillium italicum* decay of grapefruit and orange by *Debaryomyces hansenii*, NRRL Y-18314

| Citrus cultivar | Antagonist | Incubation time (days) 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| | | Percent Infection[a] | | | |
| Grapefruit (72) | NRRL Y-18314 | 3 | 3 | 4 | 6 |
| | Control | 97 | 100 | 100 | 100 |
| Orange 'Valencia' (72) | NRRL Y-18314 | 3 | 8 | 10 | 19 |
| | Control | 84 | 95 | 97 | 100 |
| Orange 'Shamouti' (72) | NRRL Y-18314 | 3 | 6 | 8 | 15 |
| | Control | 90 | 95 | 100 | 100 |

[a] Number of inoculation sites per treatment is indicated in parentheses under the cultivar's name.

TABLE III

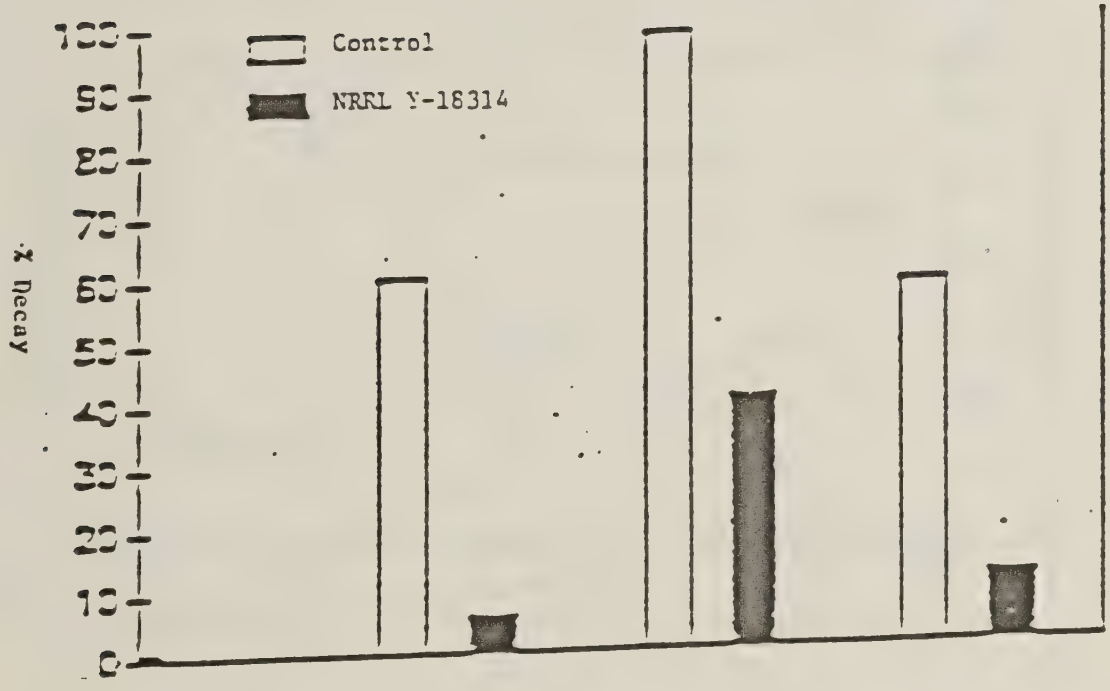
Inhibition of Geotrichum candidum decay of grapefruit and lemon by Debaryomyces hansenii, NRRL Y-18314

| Citrus cultivar | Antagonist | Incubation time (days) 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| | | Percent infection[a] | | | |
| Grapefruit (72) | NRRL Y-18314 | 3 | 3 | 8 | 9 |
| | Control | 30 | 56 | 78 | 86 |
| Lemon (30) | NRRL Y-18314 | 12 | 17 | 18 | 18 |
| | Control | 75 | 77 | 77 | 77 |

[a] Number of inoculation sites per treatment is indicated in parentheses under the cultivar's name.

TABLE IV

Inhibition of Rhizopus rot of grapes by Debaryomyces hansenii (NRRL Y-18314)[a]




Time of Decay

[a]Freshly harvested single berries or small clusters were dipped in sterile NaCl (control) or in a 48 hr culture of NRRL Y-18314 (protected) and then placed in plastic trays at room temperature for 5 days. Incidence of decay was determined by counting single berries.

TABLE V

Inhibition of Botrytis cinerea decay in apples by Debaryomces hansenii (NRRL Y-18314).

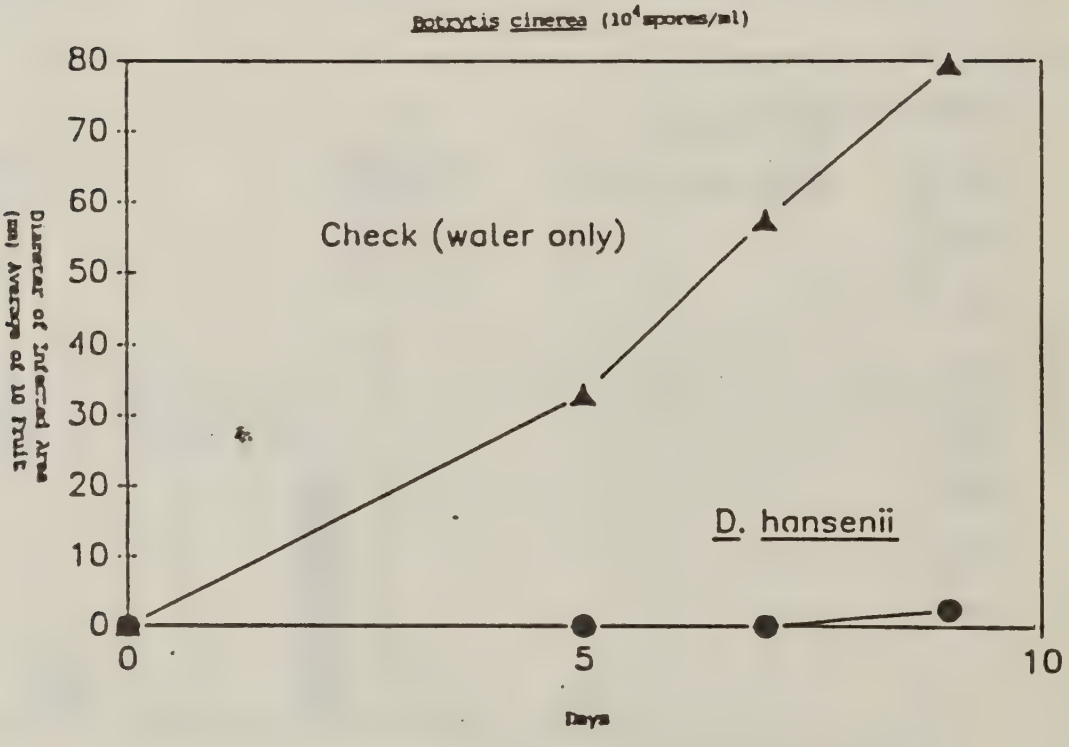


TABLE VI

Inhibition of Penicillium expansum in apples by Debaryomces hansenii (NRRL Y-18314).

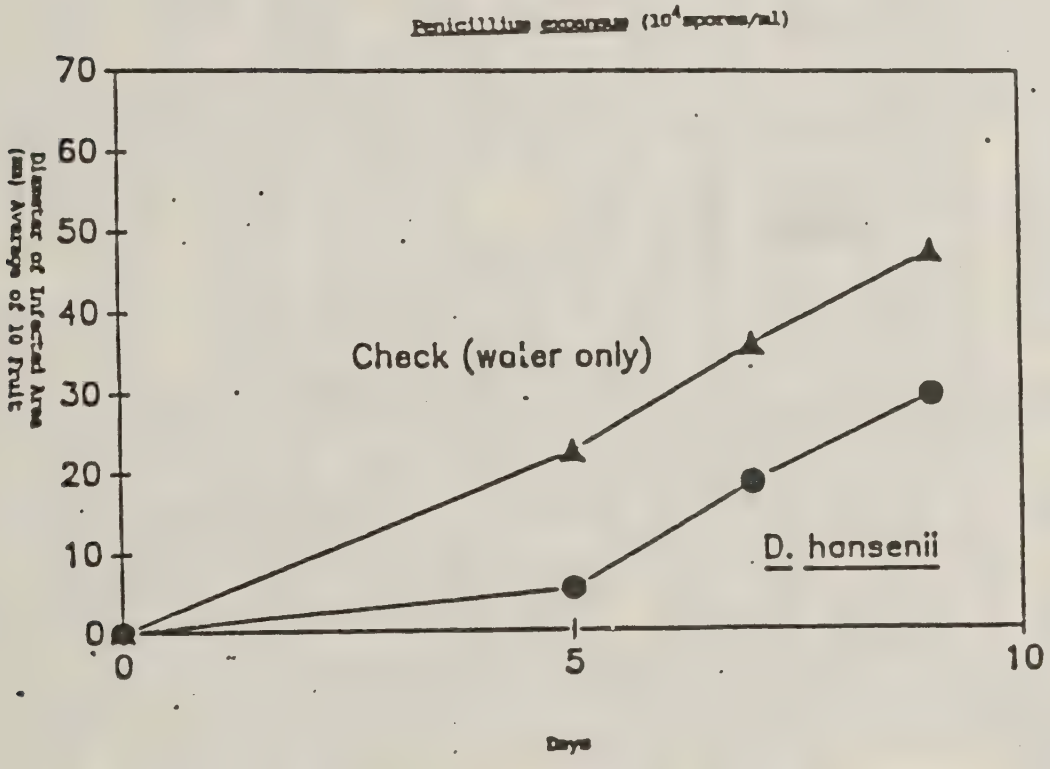
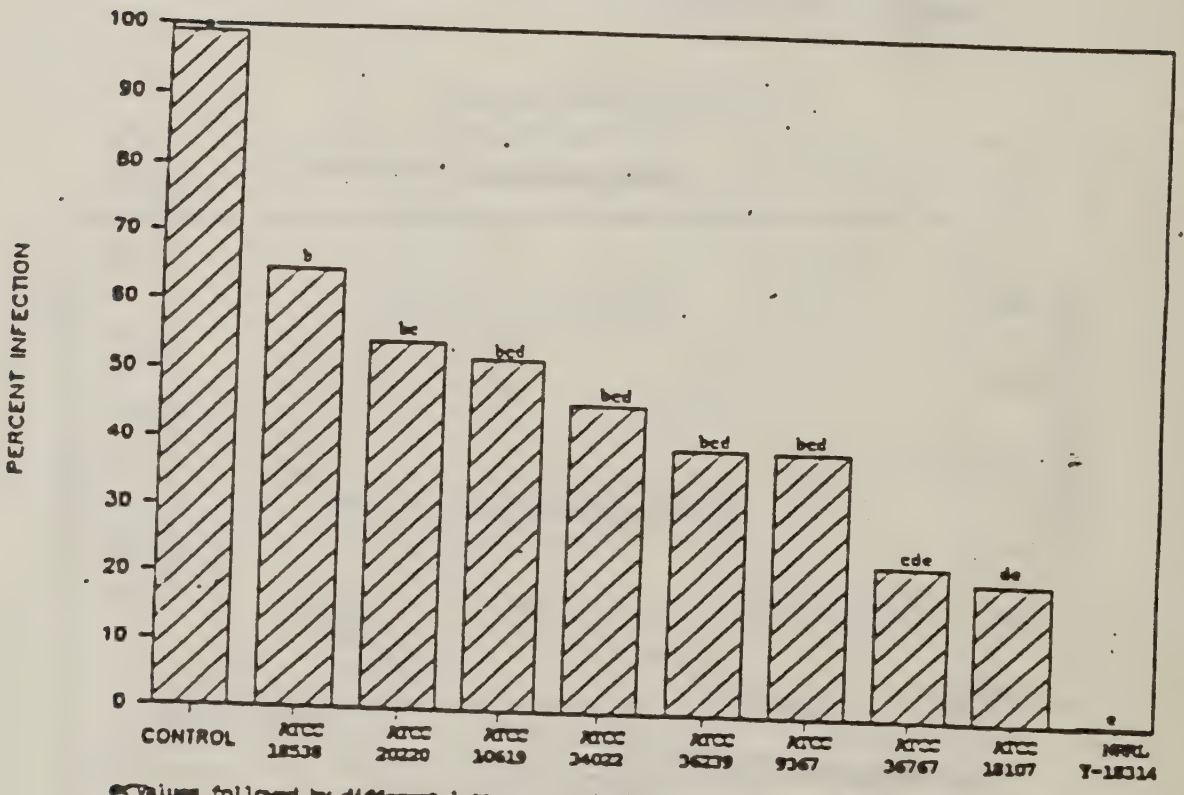


TABLE VII

Relative effectiveness of D. hansenii isolates to inhibit Penicilluim [redacted] decay on grapefruit [redacted]




[redacted]Values followed by different letters are significantly different at a level of 18 according to Duncan's Multiple Range test.

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